

Routes to Transplant Tolerance versus Rejection: The Role of Cytokines

Review

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The alloimmune response can be divided into specific junctures where critical decisions between tolerance and immunity are made which define the outcome of the transplant. At these “decision nodes” various cytokines direct alloresponsive T cells to develop either a proinflammatory response aimed at graft destruction or an immunoregulatory response facilitating graft acceptance. This review will focus on the role of these cytokines in influencing the progression of an alloimmune response leading ultimately to either allograft survival or rejection.

Introduction

Self-tolerance refers to the normal homeostatic balance in which the immune system exists without any pathologic response to self-antigens. Similarly, acquired transplant tolerance refers to the absence of a pathologic immune response to the allograft without the need for chronic, nonspecific immunosuppression. In other words, the host's immune system should be fully competent to respond adequately to further antigenic challenge without targeting the transplanted organ or tissue. Although the attainment of true transplantation tolerance (with acceptable treatment toxicity) in the clinical setting remains elusive, it can often be induced in a variety of rodent models. In these settings, intense investigation of the mechanisms involved has led to a greater understanding of the requirements for tolerance and the obstacles to be overcome if long-term allograft acceptance is to be achieved in the future.

As with other immune responses, cytokines play an integral role in alloimmunity, and the pattern of cytokine expression is central to the mechanisms which regulate the development of immune tolerance to transplanted tissues. This review will provide an overview of current concepts of alloimmunity and allotolerance with an emphasis on the part played by cytokines in these processes and discuss strategies which have been employed to exploit this knowledge as a means of inducing transplantation tolerance.

Overview of Allorecognition and Graft Rejection

Allograft rejection is primarily driven by the ability of host T cells to recognize polymorphisms encoded within

the MHC as well as multiple minor histocompatibility loci. While all components of the innate and adaptive immune systems participate in graft rejection, models in which T cell- or CD4 T cell-deficient mice indefinitely accept allografts demonstrate the paramount importance of T lymphocytes and particularly CD4 T cells in this process (Rosenberg and Singer, 1992). Host T cells can be activated in response to an allogeneic stimulus by one of two separate mechanisms (Batchelor and Lechler, 1982). Direct allorecognition defines the response of host T cells recognizing intact donor MHC molecules present on the surface of donor-derived antigen-presenting cells (APCs). In the indirect pathway of allorecognition, host T cells respond to processed donor-derived peptides bound to syngeneic MHC molecules and presented in a self-restricted manner.

Studies over the past twenty years suggest separate but complementary roles for these two pathways of allorecognition in rejection and tolerance. This does not appear to be the result of differences in the quality of the T cell response, or the cytokines elicited, but rather is based on differences in cell quantity and locale. For example, donor-derived APCs, primarily dendritic cells, are present in grafted tissues as passenger leucocytes that migrate posttransplant to host lymphoid tissues where they directly stimulate host T cells. Dendritic cells are extremely powerful activators of naive T cells, and this, coupled with the very large frequency of cells exhibiting direct alloreactivity, has led to the concept that the direct pathway of allorecognition is dominant during acute rejection (Game and Lechler, 2002; Suchin et al., 2001; Womer et al., 2001).

As migratory dendritic cells are relatively short-lived after transplantation, the role of direct alloreactivity may be temporally limited, allowing the indirect pathway to predominate in the later stages of the allograft response. Evidence in favor of this includes the findings of large numbers of indirectly reactive T cells (by limiting dilution analysis) in patients undergoing chronic rejection, but not in controls with good graft function, and the requirement for indirect allorecognition to provide B cell help for alloantibody production, a key feature of chronic rejection (Hornick et al., 2000; Pettigrew et al., 1998). Nonetheless, direct alloreactivity may not necessarily be limited to the early phases of the allograft response. Donor endothelial cells, expressing many of the same costimulatory and adhesion molecules found on dendritic cells, are able to directly activate recipient CD8 T cells (Kreisel et al., 2002). As donor endothelial cells persist for the life of the graft, they may provide a constant source of stimulation for directly alloreactive T cells. It is also important to keep in mind that the indirect pathway of allorecognition may be utilized by regulatory T cells which are required to maintain tolerance in a number of different experimental transplant models (reviewed in Cobbold and Waldmann, 1998; further discussion below).

Overview of Peripheral Tolerance

One of the most successful experimental strategies to induce allograft acceptance is to create a state of mixed

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hematopoietic chimerism (Sykes, 2001). This strategy exploits the natural homeostatic mechanisms used to create self-tolerance during T cell development in the thymus. Mixed chimerism and other strategies of central (i.e., thymic) tolerance appear to act almost exclusively via deletional mechanisms, with scant if any evidence for anergy or regulation (Nikolic and Sykes, 1997). As a result, the requirement for cytokines, as they may exist, in this process has not been investigated in depth. Thus, for the purposes of this review, we will restrict our discussion to the role of cytokines in the regulation of peripheral tolerance, i.e., strategies primarily targeted at mature T cells in secondary lymphoid organs.

It is conceptually useful to divide peripheral transplantation tolerance into two distinct but not mutually exclusive temporal phases (Li et al., 2001b). The induction phase encompasses events which occur immediately posttransplant when a large number of alloresponsive T cells are stimulated by graft antigens presented by either donor or host APCs. The development of such a robust immune response will eventually lead to acute graft rejection unless mechanisms are set in place which hold the inflammatory process in check. Thus, the induction phase of tolerance typically is characterized by depletion or ignorance of alloreactive T cells with an implicit role for a number of different cytokines.

Once this initial acute immune response has been restricted, the threat of rejection persists unless the tolerant state is maintained. The maintenance phase of allograft tolerance is an active process of T cell-mediated regulation, which suppresses the potentially injurious graft-reactive T cells. While there has long been a general acceptance of the requirement of active immunoregulatory mechanisms for the maintenance of transplantation tolerance, it is only recently that such mechanisms have been identified and characterized more closely (Wood and Sakaguchi, 2003).

Role of Cytokines in the Response to Allografts

From the above considerations, it is clear that rejection and tolerance can be considered alternative outcomes of the allograft response, i.e., host encounter with alloantigens. Once activated, CD4⁺ T cells primarily direct the progression of the response by secreting cytokines which activate, expand, and/or recruit other effector cells such as macrophages and CD8⁺ T cells, B cells, and NKT T cells. The primary consideration is defining key junctures at which alternate outcomes are favored and determining the parameters that dictate the decision ultimately made by the T cell. Here, we consider five such decision nodes and will examine the role of cytokines in these fate determinations. They are APC maturation, T helper cell expansion, T cell survival, T helper cell differentiation, and regulatory T cell homeostasis. The first four events largely take place during the induction phase of tolerance while the latter is likely of primary importance during the maintenance phase.

APC Maturation

Dendritic cells (DCs) are found in most peripheral tissues (Banchereau and Steinman, 1998) and, as noted above, migrate immediately posttransplant to regional lymph nodes where the host response to the allograft is then initiated. While the requirements for DC development

and homeostasis *in vivo* have not been clearly defined, *in vitro* systems indicate an important role for cytokines, including IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF) in particular, in promoting the development of these cells from bone marrow- and peripheral blood-derived precursors. Type-1 interferons, which are secreted from a variety of different cell types in response to inflammatory damage, can also lead to DC development *in vitro*. Infection or tissue damage (such as takes place in the context of transplantation) promote the maturation of DCs. In contrast to their immature counterparts, mature DCs can efficiently induce T cell expansion and differentiation, which has led to the emergence of a model whereby infection or inflammatory tissue damage helps provide the danger signals required for the development of an immune response. The importance of DC maturation in the context of transplantation is illustrated by studies showing that immunization of recipients with immature DCs from the same donor strain prolongs subsequent murine heart allograft survival in a donor-specific fashion (Lutz et al., 2000; O'Connell et al., 2002).

A number of microbial products such as LPS and bacterial DNA as well as necrotic cell products such as heat shock proteins have been reported to provide such an activating stimulus for DCs. These diverse stimuli are recognized by members of the Toll-like receptor family (TLRs) expressed on the surface of DCs. They promote DC maturation through activation of a variety of signaling cascades involving adaptor proteins such as MyD88 and TRAF family members leading to NF- κ B-mediated transcription (Dunne and O'Neill, 2003; Kobayashi et al., 2003). These signaling events in turn lead to the upregulation of T cell costimulatory molecules including CD40, CD80, and CD86 as well as the secretion of cytokines such as IL-12 and TNF α (Akira et al., 2001). The importance of this sequence of events in the initiation of an alloresponse has been highlighted by a recent report demonstrating the critical role of MyD88 expression in acute allograft rejection. In this report, minor antigen-mismatched allografts were not rejected when both donor and recipient mice were MyD88 deficient. Prolonged graft survival was associated with a decreased number of phenotypically mature DCs found in draining lymph nodes as well as a reduced capacity for priming of alloreactive T cells (Goldstein et al., 2003). It also has been reported that *in vitro* manipulation of DCs with low doses of GM-CSF or with IL-10 renders the cells resistant to maturation and can facilitate prolonged allograft survival (Lutz et al., 2000; Mitra et al., 1995).

Several mechanisms have been proposed by which immature DCs can promote tolerance (Figure 1) (Steinman et al., 2003). First, immature DCs constitutively express only very low (or absent) levels of T cell costimulatory molecules such as CD80/86 and CD40 as well as a relatively low level of MHC complexes on the cell surface. Moreover, immature DCs do not secrete significant levels of proinflammatory cytokines such as IL-12 and TNF α . Lack of appropriate costimulation may induce T cell anergy or apoptosis, clearly favoring tolerance. However, the relevance of this observation to *in vivo* tolerance may be questioned by the fact that, as opposed to naive T cells, activation of memory T cells is relatively independent of costimulation (Harris and

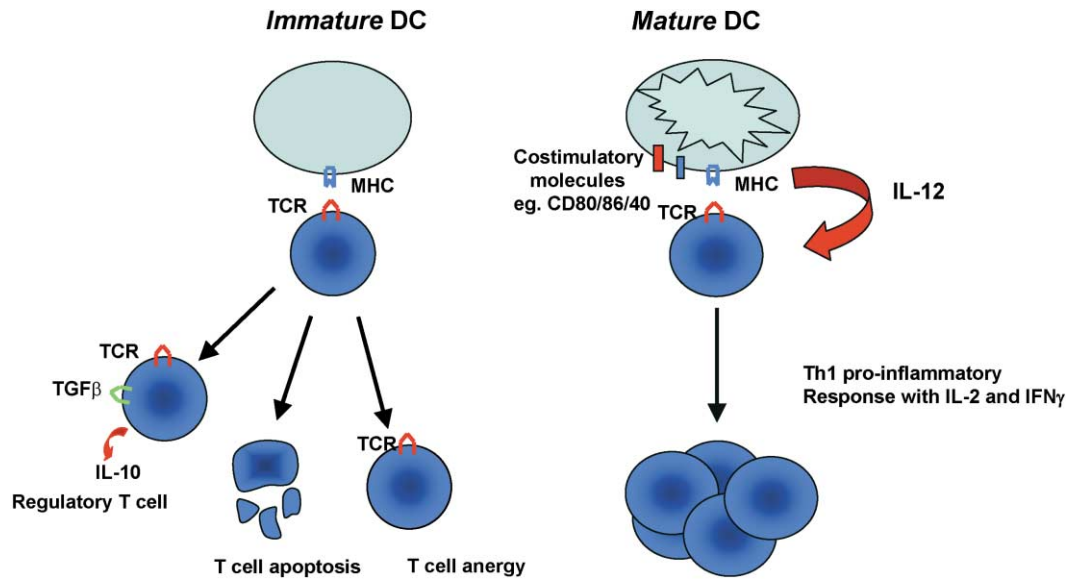


Figure 1. Dendritic Cell Maturation Determines the Fate of Responding $CD4^+$ T Cells

Immature DCs can induce a number of responses from $CD4^+$ T cells which are conducive to tolerance. These include anergy, apoptosis, or differentiation into a regulatory T cell producing immunosuppressive cytokines such as IL-10. Mature DCs, on the other hand, express elevated levels of costimulatory molecules and secrete proinflammatory cytokines such as IL-12. This allows mature DCs to efficiently prime $CD4^+$ cells to differentiate into Th1 type effector cells secreting IL-2 and IFN- γ .

Ronchese, 1999) and otherwise unmanipulated inbred mice contain substantial numbers of memory T cells (Lee et al., 1996), which can exhibit crossreactivity with alloantigens (Adams et al., 2003).

Second, and perhaps more intriguing, is the reported induction of a regulatory T cell phenotype by repeated stimulation with immature allogeneic DCs (Jonuleit et al., 2000). These induced regulatory T cells (Tr1 cells) appear to be a distinct population of cells from those Tregs which arise naturally in the thymus (e.g., $CD4^+CD25^+$ T cells, further discussion below). Similar regulatory populations have been described in models of autoimmune disease (Th3 cells), and their suppressive effect has been associated with a number of cytokines, particularly IL-10 and TGF- β (Cobbold and Waldmann, 1998; Josien et al., 1998; Roncarolo and Levings, 2000). Inducible Tregs are likely to mediate the occurrence of antigen-specific hyporesponsiveness in human T lymphocytes in vivo after injection of antigen-loaded immature DCs (Dhodapkar et al., 2001).

Although the extent of maturation of DCs undoubtedly plays an important role in determining the decision between tolerance and immunity, there also exists considerable evidence indicating that specific DC subsets may suppress T cell responses independent of their maturation state. $CD8^+$ DCs can in certain models induce tolerance of CTLs upon crosspresentation of exogenous antigen. The tolerizing potential of this DC subset appears to be related to their ability to promote deletion of the activated $CD8^+$ T cells (Heath and Carbone, 2001). There is also accumulating evidence for the existence of a discrete population of DCs expressing the enzyme indoleamine 2,3-dioxygenase (IDO) which constitutes the rate-limiting step in the catabolism of tryptophan. Several reports have now documented the role of IDO in

tolerance to allogeneic fetuses. While it was originally postulated that IDO acted by depriving T cells of a requisite growth factor (tryptophan) (Mellor and Munn, 1999), more recent studies indicate that it is the tryptophan metabolites themselves which exert a cytotoxic effect on T cells (Frumento et al., 2002; Terness et al., 2002). Stimulation of mature IDO expressing DCs with IFN- γ results in downregulation of this enzyme, an effect which is inhibited by the presence of IL-10 (Munn et al., 2002).

T Helper Cell Expansion

An obvious role for cytokines in allogeneic responses, and indeed their first identified role for T cells, is to promote T cell proliferation. Limiting the expansion of effector T cells is an integral part to any approach toward promoting tolerance, but until recently, it has been unclear which cytokines, if any, might be specifically and, if possible, nonredundantly important for T cell expansion in vivo. The cytokines which can act as T cell growth factors are primarily those whose multimeric receptor complexes contain the common γ (γ c) chain, e.g., IL-2, IL-4, IL-7, IL-15, and IL-21. IL-2 was the first such cytokine identified. Its discovery and the production of antibodies against the α chain of the IL-2R (CD25) prompted studies which demonstrated that anti-CD25 mAbs could induce transplantation tolerance (Kupiec-Weglinski et al., 1987; Reed et al., 1989). Initially, it was assumed that this was mediated by blockade of clonal expansion of pathogenic alloreactive T cells. However, a variety of lines of evidence subsequently indicated a far more complex situation. First, calcineurin inhibitors such as cyclosporine and tacrolimus are potent inhibitors of IL-2 production, yet they fail to induce tolerance in mice and humans. Second, IL-2-deficient, as well as IL-2/IL-4 doubly deficient mice readily reject heart and islet allografts (Li et al., 1998a). Indeed, IL-2-deficient animals

are in fact difficult to tolerize (see below). Third, it is clear that IL-2 is not the only, or perhaps even the most, important *in vivo* growth factor for activated T cells. While the use of IL-2 by T cells may predominate *in vitro* where T cells themselves are the source of cytokines, *in vivo* T cell proliferation appears to be much more dependent on IL-15, which is produced by diverse cell types such as APCs and endothelial cells (Li et al., 2001a).

So how does anti-CD25 work? It is important to remember that in fact there is a hierarchy of tolerance susceptibility in murine models: liver > kidney > heart > islet >> skin. Anti-CD25 by itself works only in more permissive models. A recent study may provide some insight into this. Here, it was found that anti-CD25 lysed activated CD25⁺ effector T cells, while also targeting CD25⁺ Tregs (Zheng et al., 2003). In contrast, use of an agonistic IL-2/Fc fusion protein selectively targeted CD25⁺ effector T cells while sparing CD25⁺ Tregs and promoting allograft survival in a very stringent model. This approach exploits the ability of IL-2 to induce activation-induced cell death (AICD) in effector T cells while promoting the survival of CD25⁺ Tregs.

T Cell Survival

In the last several years, a very complex story has emerged regarding the ability of cytokines to alternately promote T cell survival and death and the role these functions play in the net effect of cytokines on transplantation tolerance (Figure 2). Particular attention has focused on IL-2, and much of this evidence stems from investigations in mice, which are deficient in expression of IL-2 or the α chain of the IL-2R. Such mice have a severe lymphoproliferative disorder caused by an apparent failure to clear activated T cells from the periphery through AICD (Sadlack et al., 1995; Willerford et al., 1995). T cells from IL-2^{-/-} mice were observed to be insensitive to Fas-mediated apoptosis *in vitro* leading to the hypothesis that a major function of IL-2R signaling *in vivo* is to prime activated T cells for Fas-induced apoptosis (Refaeli et al., 1998). At present, it does not appear that any of the other γ chain cytokines can also act as a feedback regulator of clonal expansion similar to IL-2. Interestingly, the other major cytokine associated with an inflammatory immune response, IFN- γ , also has an important role in regulating T cell expansion, as revealed by studies in IFN- γ -deficient mice, which display enhanced proliferation in response to allogeneic stimulation *in vitro* when compared to their wild-type counterparts (Konieczny et al., 1998).

However, while IL-2 has the ability to prime T cells to ultimately undergo AICD, its initial role in the immune response is actually to promote T cell survival, primarily by upregulation of the antiapoptotic gene Bcl-2 (Mueller et al., 1996; Van Parijs et al., 1999). Expression of Bcl-2 and the related gene Bcl-xL, which is upregulated through CD28 costimulation (Boise et al., 1995), enables activated cycling T cells to survive in cytokine-depleted environments. Thus, the net effects of IL-2 are both time and context dependent. Early in the immune response, and in the face of limiting cytokines, IL-2 promotes T cell growth and survival. Later in the immune response, and in the face of continued antigenic stimulation, IL-2 promotes cell death.

In addition to IL-2, other cytokines such as IL-15, IL-4,

and IL-7 have been reported to play a role in activated T cell survival *in vivo* and as such can directly regulate the clonal size of alloresponsive T cells (Vella et al., 1998). The overlapping effects of these cytokines are thought to be linked to the shared expression of a common γ chain by their respective receptors. Redundancy of function between these related cytokines may help explain why strategies which target expression of a single family member have proved largely unsuccessful in inducing allospecific tolerance (Li et al., 1998a; Steiger et al., 1995). In support of this hypothesis, recent studies have demonstrated that blockade of γ chain signaling induces the long term survival of islet allografts while inducing T cell apoptosis (Li et al., 2000). Similarly, administration of a humanized monoclonal antibody, which blocks signaling through the IL-2R β chain, prolongs renal allograft survival in cynomolgus monkeys (Tinubu et al., 1994). As the IL-2R β chain is shared by the receptors for both IL-2 and IL-15, this agent inhibits both the discrete and overlapping effects of these cytokines on T cell-mediated immune responses (Waldmann et al., 2001). These observations indicate that it may be more efficient to target multiple cytokines over individual cytokine pathways as a means of inducing tolerance.

The question of T cell survival may be particularly important for transplantation tolerance. Recently, we and others have demonstrated the necessity of a reduction in alloresponsive T cell clone size through deletion as a prerequisite for achieving tolerance (Li et al., 1999; Wells et al., 1999; Dai et al., 1998). In these studies, we found that mice whose T cells were defective in undergoing either AICD (IL-2-deficient mice) or in undergoing apoptosis in response to cytokine withdrawal (Bcl-xL transgenic recipients) resisted tolerance induction to MHC mismatched allografts by costimulatory blockade. Furthermore, calcineurin inhibition, which blocks the induction of apoptosis following T cell activation, antagonized the ability of costimulatory blockade to induce tolerance. Conversely, rapamycin, which potentiates AICD induced through costimulatory blockade, enhanced the tolerizing potential of this approach in highly resistant models such as skin. The importance of cell death in the induction of tolerance further explains why both IL-2 and IFN- γ , proinflammatory cytokines which might be considered a barrier to tolerance induction, can in fact, through their ability to promote T cell apoptosis, also facilitate rather than impede allograft acceptance. These observations suggest that these so-called "proinflammatory" cytokines play an additional, if not predominantly limiting, role in the context of an *in vivo* alloimmune reaction (Figure 2).

During the course of a normal T cell immune response, initiation is followed by clonal expansion and a subsequent large-scale apoptotic cascade, which memory cells escape. This is likely due to an inherent survival advantage of memory T cells over their effector counterparts, and as a consequence memory cells may evade tolerizing strategies, which induce the deletion of alloreactive T cells during the induction phase. While the requirements for the generation of CD4⁺ T cell memory remain largely undefined, it is increasingly evident that IL-15 plays a central role in the generation and survival of CD8⁺ memory cells (Figure 2) (Schluns and Lefrancois, 2003). In contrast, IL-2 has been reported to have little

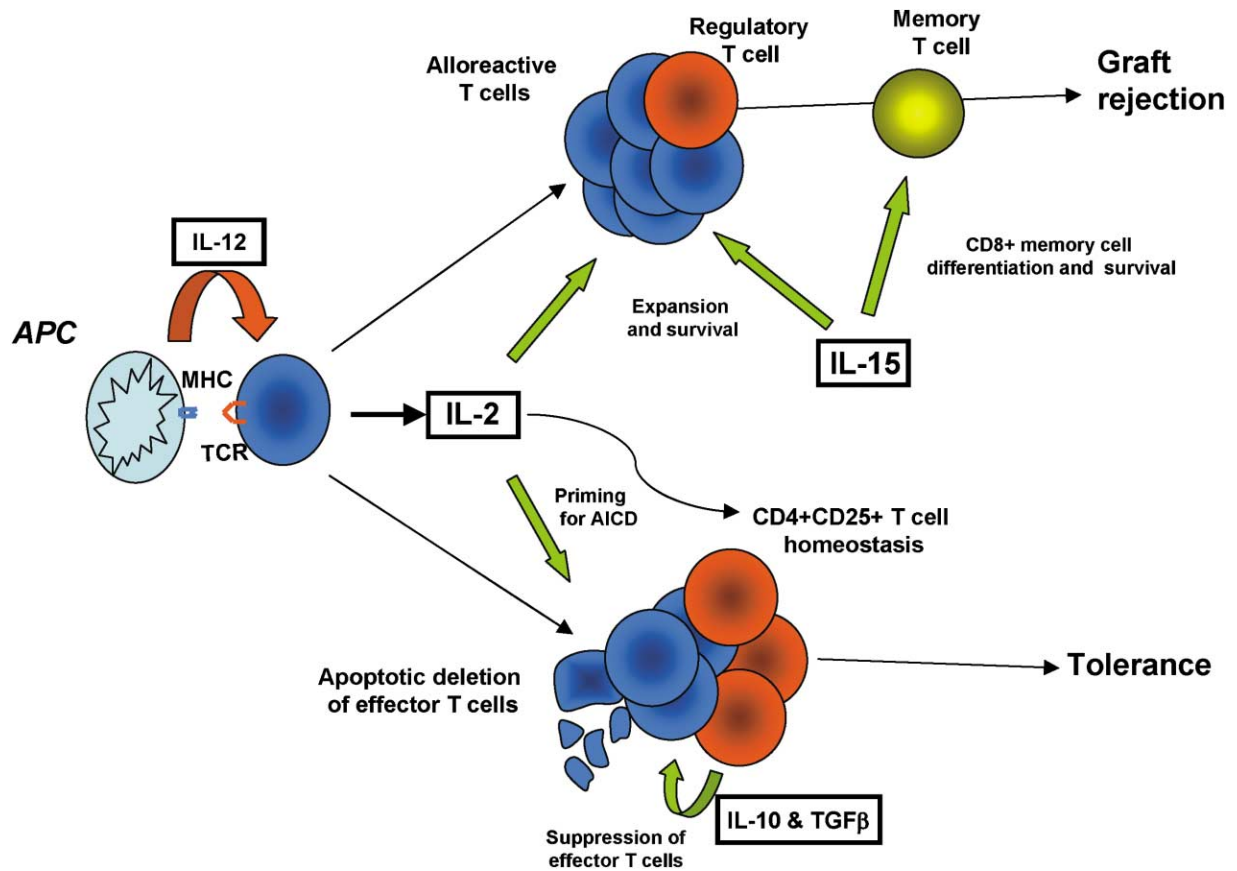


Figure 2. Role of Cytokines at Major Decision Nodes between Allograft Tolerance and Rejection

Cytokines play a pivotal role in the development of both transplant tolerance and rejection by regulating critical points during the generation of an allospecific immune response. Proinflammatory cytokines such as IL-12 and IL-15 appear to exclusively promote graft rejection through directing T effector differentiation or promoting expansion, survival, and memory development, respectively. Conversely, immunosuppressive cytokines such as IL-10 and TGF β can promote tolerance through mediating regulatory T cell function. IL-2 can either be tolerogenic (priming for AICD and regulating Treg homeostasis) or promote rejection (effector T cell expansion and survival). The pleiotropic effects of IL-2 appear to be temporally and contextually dependent.

or no effect on CD8⁺ memory cell homeostasis, indicating that this function is specific to IL-15 and not shared by related cytokines (Dai et al., 2000). An important feature of memory T cells is their decreased requirement for costimulation to induce optimal responses. In fact, costimulatory blockade has been demonstrated to be ineffective in preventing allograft rejection in certain models and this resistance may be mediated by CD8⁺ memory T cells (Valujskikh et al., 2002; Zhai et al., 2002). Other studies have demonstrated that antagonism of IL-15 signaling and/or lysis of IL-15R⁺ cells can promote allospecific tolerance in models which are resistant to the effects of costimulatory blockade (Ferrari-Lacraz et al., 2001; Smith et al., 2000). However, it has not been conclusively proven that all of these effects are mediated specifically through memory cells, as primary CD8⁺ T cells also have a reduced requirement for costimulation when compared to their CD4⁺ counterparts (Newell et al., 1999).

T Helper Cell Differentiation

Th2 responses are often observed in the context of long-term allograft acceptance and/or tolerance. This initially led to the proposal that Th2 immune deviation was caus-

ally linked to transplant tolerance; however, many subsequent investigations indicate that the situation is somewhat more complex. For example, in most circumstances, deliberate skewing of alloimmunity toward Th2 responses either through antagonism of IL-12 signaling or in vivo administration of stable Th2 cytokines still results in rejection of MHC mismatched grafts (Li et al., 1998b; Piccotti et al., 1996; Zheng et al., 1995). However, redirecting a Th1 response toward a Th2 response is by itself sufficient to prevent graft rejection across selected minor histocompatibility barriers (Li et al., 1998b). We have interpreted this in the context of the size of the alloreactive T cell pool, being much larger in across MHC barriers than across minor histocompatibility differences (Kishimoto et al., 2002). Consistent with an important role for pool size, while costimulatory blockade cannot induce long-term MHC mismatched allograft survival in models where T cell apoptosis is impaired (see above), CTLA4Ig is very effective at inducing long-term allograft survival across mH barriers in these same models (IL-2- or IFN- γ -deficient recipients or Bcl-xL-transgenic recipients). Thus, the relative ease of creating a tolerant state, and the requirements to do so, may

differ depending on quantitative issues (e.g., the number of alloreactive cells). Nonetheless, other reports indicate that Th2 alloreactive clones can be effective mediators of graft rejection (Barbara et al., 2000) and murine strain specific differences in the Th1 versus Th2 dominance of allograft rejection have been reported, with either type of response culminating in graft destruction (Wang et al., 2003). While these studies make it clear that Th2 responses can be directed at allograft rejection, the ultimate role of Th2 cytokines in graft rejection versus tolerance remains unresolved. Any such role is undoubtedly related to a number of parameters including the size of the alloreactive T cell repertoire, the extent and types of antigenic disparities between donor and recipient, and the tissue being transplanted, as well as the background genes of the recipient which influence the nature of the allograft response.

There is, however, one well-described system in which immune deviation can induce allograft tolerance and whose mechanism is understood at a cellular level. The term anterior chamber-associated immune deviation (ACAID) was coined to describe a naturally occurring phenomenon which facilitates the transplantation of MHC-mismatched corneas with the absence of a proinflammatory T cell response (Streilein et al., 2002). ACAID occurs as a result of a number of factors, which combine to promote the development of a regulatory response by APCs normally resident within the anterior chamber of the eye. APCs at this site are phenotypically modulated by the high levels of constitutive expression of TGF- β in the intraocular microenvironment, leading to the downregulation of T cell costimulatory factors such as CD40 and a failure to secrete IL-12. After antigen capture these APCs are mobilized to leave this environment and migrate to the spleen. Here the APCs continue to secrete immunosuppressive cytokines, in particular, TGF- β and IFN α/β , and upon initial interaction with NK T cells attract antigen-specific CD4⁺ and CD8⁺ T cells. The net effect of such phenotypic alterations in antigen presentation is the development of an immunoregulatory T cell response in place of a Th1-type response. Immunoregulatory T cells are induced to produce cytokines such as TGF β and IL-10 and disseminate from the spleen to contribute to the maintenance of tolerance against the antigen in question.

Regulatory T Cell Homeostasis and Function

While deletional events during the induction phase of transplantation tolerance prevent early rejection and facilitate graft acceptance, an active process of immune regulation is required to maintain host tolerance to transplanted tissues over a prolonged time period.

Initial observations which established that tolerance to alloantigens is transferable to a naive animal by T cells provided evidence of the existence of immunoregulatory mechanisms within the host's T cell repertoire (Qin et al., 1993). The existence of distinct T cell subsets, which function to restrict the expansion of effector T cells is now widely accepted, and the identification and mechanism of action of such regulatory T cell (Treg) subsets remain areas of intense investigation. While the majority of Treg subsets defined thus far develop within the CD4⁺ population, regulatory activity is by no means exclusively restricted to CD4⁺ T cells. Indeed, regulatory activity has also been demonstrated in CD8⁺ as well as in

TCR⁺CD4⁻CD8⁻ T cells after transplantation (Colovai et al., 2000; Zhang et al., 2001). Tregs have been reported to have a variety of actions on immune effector cells, most notably the inhibition of proliferation and cytokine production. However, the precise mechanisms of immunosuppression by Treg subsets have yet to be defined, and it is becoming increasingly clear that various cytokines play an integral role in the development, homeostasis, and function of these cells.

Most recent studies on Tregs have focused on a naturally occurring subset of CD4⁺ T cells which constitutively express the IL-2R α chain (CD25). While CD4⁺CD25⁺ Tregs were originally characterized as being important in the maintenance of self-tolerance (Takahashi et al., 1998), they are also recognized to play a role in many models of allograft tolerance. The suppressive effect of these cells appears to be cell contact dependent, and there is evidence to suggest that Treg-mediated production of IL-10 and cell surface expression of TGF β may play an important role in vivo. Inhibitory antibodies against both IL-10 and its cognate receptor have been shown to inhibit the suppressive effects of Treg cells in skin allograft models (Hara et al., 2001; Kingsley et al., 2002). However, a defined role for IL-10 in transplantation tolerance remains controversial, perhaps reflecting both the immunostimulatory and immunosuppressive properties of this cytokine (Figure 4). Accordingly, while some studies reveal the beneficial effects of IL-10 signaling in enhancing graft survival many others suggest neutral or even detrimental effects (Moore et al., 2001). Interestingly, a homolog of IL-10 expressed in many viral genomes appears to retain the immunosuppressive properties of this cytokine without the capacity for immunostimulation observed with endogenous IL-10. Indeed, studies involving ectopic expression of viral IL-10 within the allograft have had considerably more success in prolonging graft survival than models which manipulate endogenous IL-10 (Bromberg, 1995; DeBruyne et al., 1998; Wang et al., 1999). The observation that Treg cells express a cell surface bound isoform of TGF β has led to the hypothesis that it may be the mechanism through which cell contact-mediated suppression occurs. Indeed, a role for TGF β in mediating suppression by Tregs has been described in a number of allograft models (Jonuleit et al., 2002; Josien et al., 1998). However, it is noteworthy that functional CD4⁺CD25⁺ Tregs are present in mice with deficiencies in both IL-10 and TGF β , perhaps indicating that these cytokines alone are not solely responsible for suppression (Piccirillo et al., 2002; Suri-Payer and Cantor, 2001). It should also be noted that most reported data would also be consistent with a model in which cytokines such as IL-10 and TGF β are not necessarily direct products of the Tregs, but may be induced by Tregs in other target populations.

Along those lines, an interesting feature of regulatory T cells in the context of allograft transplantation is an apparent ability to promote the development of an immunosuppressive phenotype in otherwise nonregulatory T cells. Furthermore, an allounresponsive state can be generated without subjecting the host to the entire range of donor graft antigens. Preexposure to a single antigen expressed within the graft can be sufficient (Madsen et al., 1988). This linked suppression appears

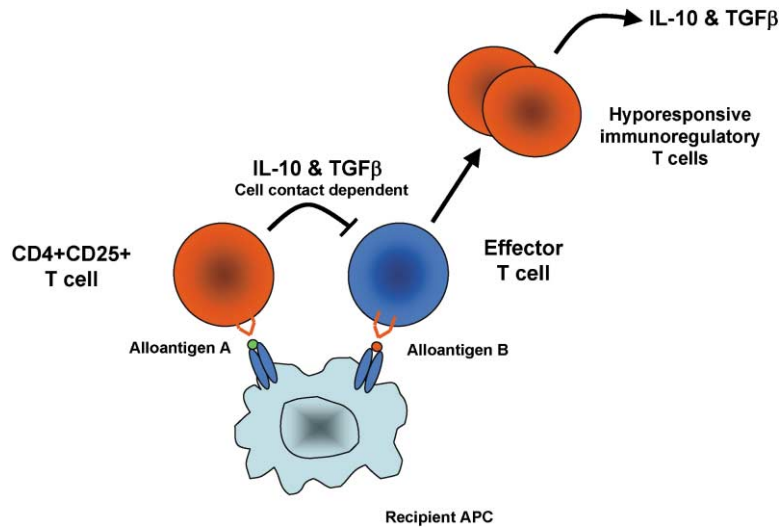


Figure 3. The Phenomenon of Linked Suppression

Linked suppression refers the induction of tolerance to a third party alloantigen B by regulatory T cells recognizing alloantigen A when both antigens are processed and presented by the same antigen-presenting cell. This effect is mediated at least in part by the secretion of cytokines such as TGF β and IL-10 by the regulatory T cells which cause the effector cell recognizing alloantigen B to become hyporesponsive and perhaps develop a regulatory phenotype (infectious tolerance).

to act via inducing new T cells which enter the host repertoire after transplantation to develop a regulatory rather than effector phenotype (Figure 3). The exact mechanism by which linked suppression occurs has not been defined; however, it has been reported that increased Th2 type cytokines (IL-4 and IL-10) are present at the graft site in models where tolerance is achieved through such mechanisms (Onodera et al., 1997). IL-10 and TGF β have also been implicated in the suppressive function of these induced Treg subsets (Dieckmann et al., 2002; Jonuleit et al., 2002).

In addition to the ability of cytokines to mediate suppressive functions of regulatory T cells, there is mounting evidence that cytokines, in particular IL-2, play a critical role in the development and homeostasis of Treg cells (Figure 2). CD4⁺CD25⁺ T cells are not detectable in IL-2^{-/-} mice, which suffer from a severe lymphoproliferative disorder and autoimmune disease. Significantly, the generation of IL-2^{-/-} mice on a TCR-transgenic background blunts the progression of autoimmune disease, presumably because of the lack of autoreactive T cells generated naturally in the thymus (Wolf et al., 2001). Malek et al. have demonstrated the requirement of IL-2R signaling through the β chain of the receptor for the development of CD4⁺CD25⁺ Tregs in vivo (Malek et al., 2002), and it has also been reported that IL-2 contributes to the survival of Tregs in the periphery (Almeida et al., 2002; Murakami et al., 2002). A characteristic feature of CD4⁺CD25⁺ Tregs is their hypoproliferative response to IL-2 stimulation in vitro, despite constitutive expression of all three chains of the IL-2R. Proliferation of Tregs can be induced in vitro upon stimulation through either the TCR or TNFR superfamily member GITR when either stimulus is used in combination with IL-2 (McHugh et al., 2002). While recent studies have highlighted the requirement for TCR-derived signals to induce Treg expansion in vivo (Walker et al., 2003), it is highly likely that such a stimulus alone is not sufficient to drive Treg expansion and that a secondary cytokine signal is necessary. Whether IL-2 contributes to cell cycle progression or simply functions to maintain Treg viability thus contributing to efficient expansion, driven by another cytokine (e.g., IL-15), remains unclear.

Cytokines as Potential Therapeutic Targets

Arguably, the greatest single barrier to the induction of allospecific tolerance is the extraordinarily large number of responding T cells with direct allospecificity. Mounting evidence indicates that in order for tolerance to be induced an initial wave of deletion of effector T cells is required. This creates a more level playing field on which regulatory T cells can suppress the now reduced number of alloreactive cells available and primed for graft destruction.

At present, the most effective single therapeutic agents which prolong graft survival are calcineurin inhibitors, such as cyclosporine A and tacrolimus. The primary effect of these agents is to broadly block T cell cytokine gene transcription by inhibiting activation of NFAT transcription factors. However, cytokines both promote and terminate immune responses. This is well demonstrated by the findings that calcineurin inhibitors abrogate tolerance induction by costimulatory blockade, in association with inhibition of T cell apoptosis (Li et al., 1999).

It is likely that tolerance-opposing effects of calcineurin inhibitors are multifactorial. For example, by blocking the production of IL-2, these agents may prevent T cell priming for AICD which might otherwise occur during the natural evolution of an immune response or as a deliberate effect by other pharmacological or biological agents. The inability to tolerize IL-2-deficient mice by costimulatory blockade is consistent with this concept. In addition, it is likely that agents which target costimulatory molecules, such as CTLA4Ig or anti-CD154, are effective in inducing peripheral tolerance by targeting activated T cells. Inhibition of costimulatory pathways decreases secretion of prosurvival cytokines rendering activated T cells susceptible to apoptosis due to cytokine withdrawal. By preventing the early stages of T cell activation, calcineurin inhibitors may prevent the dependency on cytokines that T cells develop upon cell cycle entry. As a result, costimulatory blockade does not result in T cell apoptosis in the context of calcineurin inhibition (Li et al., 1999).

An additional major problem with the use of nonspecific immunosuppressive agents is the diverging effects

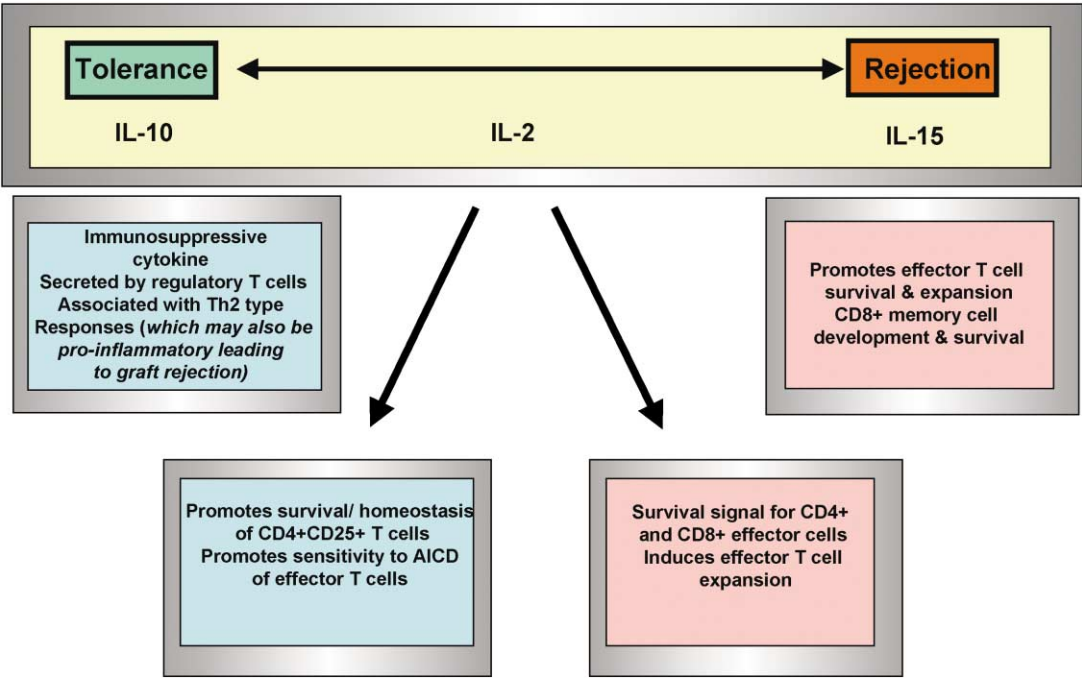


Figure 4. Cytokines Regulate the Polarity of the Immune Response toward the Transplant

Many studies demonstrate that IL-10 plays a role in the induction of T cell tolerance due to its immunosuppressive function; however, other reports indicate that it may also act in an immunostimulatory fashion to promote rejection. On the other hand, it is believed that IL-15 promotes the development of a solely proinflammatory T cell response leading to allograft rejection. IL-2 can induce both tolerizing and proinflammatory effects, depending on the phase and context of the response, demonstrating its critical role in determining the fate of the allograft.

of such regimens on different T cell subsets such as Tregs. As IL-2 is critical for Treg development and survival in the periphery, any therapy which inhibits IL-2 production is likely to have a detrimental effect on the maintenance of a regulatory T cell pool. Thus, while calcineurin inhibition may be effective in preventing the progression of an alloreactive T cell response during the acute phase after allograft transplantation, it may also prevent the equally important maintenance of allograft tolerance through its negative effect on Treg populations (Figure 4).

A more rewarding approach may be to target cytokine signaling distal to receptor engagement, when selected effects of intracellular cytokine signaling may be specifically blocked while allowing those pathways which are important to tolerance induction and maintenance to remain intact. Rapamycin is one such agent which blocks IL-2-mediated proliferation without preventing

the induction of susceptibility to AICD of activated effector T cells. As a result, when administered in combination with strategies to induce costimulatory blockade, rapamycin is permissive (if not additive) for promoting cell death and inducing peripheral tolerance (Li et al., 1999). The effects of selective inhibition of IL-2-dependent signaling with rapamycin on Treg cells are largely undefined. However, it is possible that they may differ from the effects observed in activated effector T cells given that CD4⁺CD25⁺ T cells have somewhat distinct responses to IL-2R stimulation.

The redundancy of cytokine functions, where any of several cytokines can promote T cell growth and survival, as well as the pleiotropic nature of specific cytokines, which can have both time- and context-dependent enhancing and terminating effects on immune responses, has made it very difficult to target specific individual cytokines or their receptors to promote trans-

Table 1. Roles of Major Cytokines in the Development of Transplant Tolerance or Rejection

Cytokine	Tolerance	Rejection	Reference
IL-2	+	+	Wells et al., 1999; Malek et al., 2002; Refaeli et al., 1998
IFN- γ	+	+	Konieczny et al., 1998; Akira et al., 2001
IL-10	+	+	Moore et al., 2001; Bromberg, 1995
TGF β	+	-	Jonuleit et al., 2002
IL-15	-	+	Ferrari-Lacraz et al., 2001
IL-12	-	+	Banchereau and Steinman, 1998
IL-4	+	+	Onodera et al., 1997; Vella et al., 1998

The role of cytokines in transplantation tolerance and rejection is based on the reported influence on T cell-mediated responses as well as investigation of their respective roles in models of transplantation.

plantation tolerance (Table 1). Our own belief is that the key to generating and sustaining tolerance is reducing the overall number of alloreactive T cells while allowing for the development of immunoregulatory mechanisms, some of which are likely to involve Th2 cytokines. In this context, the emergence of Th2 responses may be viewed as permissive for tolerance. A recent report demonstrating tolerance induction in a very stringent model through the combined blockade of IL-15 signals (critical for T cell proliferation *in vivo*) and the use of an agonistic IL-2/Fc fusion protein to promote the selective lysis of nonregulatory CD25⁺ T cells is just one example of how this may be achieved (Zheng et al., 2003). Clearly, a greater understanding of the specific roles these cytokines play in the context of allograft transplantation may allow other specific targeting for therapeutic strategies in the future.

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